ANALYSIS OF EPITOPE SPECIFICITY OF MONOCLONAL ANTIBODIES BY COUNTERFLOW ISOTACHOPHORESIS ON NITROCELLULOSE MEMBRANES

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When working with monoclonal antibodies (MCAB) to an individual antigen (AG) it is necessary to determine that epitope specificity, i.e., their targeting toward the same or different determinants. Competitive analysis of MCAB is usually used for this purpose, by solid-phase radioimmunoassay [4], for which purified AG and radioactive labeling of each of the MCAB to be compared are required. In the investigation described below it is shown that epitope specificity of MCAB can be analyzed by counterflow isotachophoresis (ITP) on a nitrocellulose membrane (NCM) [1] on a model of MCAB to human (HAFP) and mouse (MAFP) α-fetoprotein (AFP). The method does not require purified AG or MCAB, or their radioactive labeling, it can be performed automatically, and requires only the simplest electrophoretic equipment.

Rat MCAB to MAFP and mouse MCAB to HAFP were used. Rat MCAB were obtained as described previously [6]. Culture media (CM) of three hybridomas (D_6, G_4, H_6), both initial and concentrated tenfold by precipitation with $(NH_4)_2SO_4$ (0.33 of saturation), were used for analysis. MCAB to HAFP were obtained by fusing the splenocytes of a mouse immunized with a purified HAFP preparation and myeloma X63-Ag8.653 cells. MCAB of three cell lines (D_{10}, D_8, E_3) in mouse ascites fluid were used. Rabbit antiserum of MAFP (anti-MAFP) was obtained as described previously [2] and neonatal mouse serum (NMS) was used as the MAFP preparation. A commercial immunodiagnostic serum for primary carcinoma of the liver (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR), including rabbit anti-HAFP and AG, containing 50 μg HAFP in 1 ml and with a large excess of serum albumin, was used as the system for determining HAFP. Mouse MCAB to rat L_K IgG (rat anti-L_K containing 3 mg of antibodies in 1 ml [5] and rat IgG (10 mg/ml), generously provided by A. Yu. Rudenskii (All-Union Research Institute of Genetics, Main Administration of the Microbiological Industry of the USSR), were used. Fab'-fragments of donkey antiserum to rabbit IgG, conjugated with horseradish peroxidase (P) (Fab'-P), were obtained by V. S. Poltorandina by the method in [8].

To abolish cross reactions between reagents used in the analysis, embryonic calf serum (ECS) was added to the anti-MAFP, and rat IgG and ECS were added to the Fab'-P in concentrations chosen beforehand.

The analyses were carried out on NCM with a pore diameter of 0.45 μ (Schleicher und Schüll, West Germany, or Bio-Rad, USA). Depending on the amount of MCAB for testing, NCM 2-9 cm wide and 17 cm long were used. The zone of fixed immunological reagents was located in the middle part of the film (Fig. 1). The points of application and fixation of MCAB were marked out, and reservoirs (R) formed for the reagents on NCM moistened in buffered physiological saline (BPS). Nonspecific binding of NCM with the test proteins was prevented by clogging the pores of the NCM with 2% egg albumin or a 10% solution of "Bona" milk mixture (Finland) for 1 h.

Counterflow ITP was carried out in the following buffer system: anode (leading) 0.06 M Tris-HCl, pH 6.7, and cathode (closing) 0.012 M Tris-β-analine, pH 8.6, prepared from analytically pure reagents ("Serva," West Germany). The technique of ITP on NCM and the method of developing the precipitates were described previously [7]. As the substrate for P, 3,3'-diaminobenzidine·4HCl (0.05%) and H_2O_2 (0.01%) were used.

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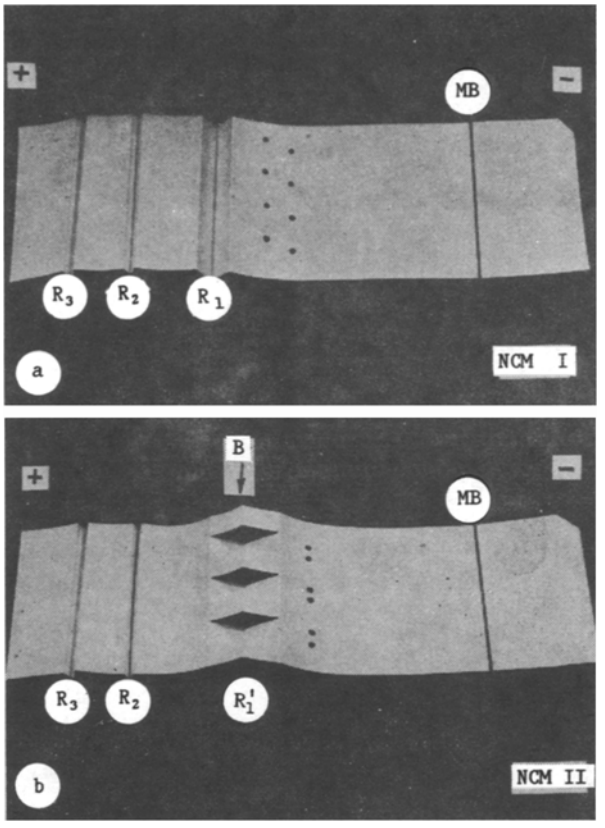

Fig. 1. Diagram showing scheme of experiment. a and b) NCM I and NCM II — two strips of NCM. Data indicate site of fixation of MCAB; R_1, R_2, R_3) reservoirs (folds), R'_1) reservoir cut into sections, B) bridge.

Electrophoretically neutral cyancobalamine (vitamin B12) was used as the electroendosmotic flow (EEF) reference substance.

The NCM were prepared for the experiment as follows. MCAB to HAFP (or MAFP) were applied in a volume 1-3 µl either directly to the NCM, or to an immunosorbent prepared on NCM, which was used for the analysis of preparations with a low content of antibodies: rat MCAB to MAFP in CM. To obtain the immunosorbent, rat anti-I_K in the chosen working dilution was applied in a volume of 1-3 µl directly to NCM. Films with spots (dots) of rat anti-L_K fixed on them were clogged with inert protein, washed in BPS, and on their anode part, which was free from dots, R for immunoreagents were formed at a distance of 1.5-2 cm apart, so that these reagents in the course of ITP were shifted by the EEF toward the cathode in isolated, nonoverlapping zones (Fig. 1). If under these circumstances the reagent had to interact with all the dots at once, R for it were made in the form of a fold, crossing the whole width of the NCM (Fig. 1a). In cases when the dots of MCAB had to be treated with different reagents, the fold was cut into sections 0.5-1.5 cm long, leaving bridges of NCM 0.5 cm wide between them (Fig. 1b). These bridges ensure uniformity of the electric field and of EEF. The NCM were washed in the leading buffer in the presence of traces of bromphenol blue (BPB) and placed in the electrophoresis apparatus.

With a voltage of 50 V on NCM a moving boundary (MB) of ions was formed, and was clearly visible because of concentration of BPB on it (Fig. 1). When MB passed into the steady state, HAFP or MAFP was introduced into R_1, the corresponding rabbit antiserum into R_2, and Fab'-P into R_3, and vitamin B_{12} was applied alongside R_3 (on the anode side). Electrophoresis was carried out overnight at a voltage of 30 V. The experiment was stopped when B_{12} crossed MB. However, with this order of addition of the reagents, a large part of the Fab'-P was lost on rabbit IgG–bR_2 aggregates. We therefore began to introduce Fab'-P into the experiment much later than the first two reagents, when free rabbit IgG had left the reaction zone and were approaching MB, as shown by the B_{12}, which was located alongside R_2 in these experiments. In that case Fab'-P was introduced into R_1, and electrophoresis carried out at 80-100 V. At the

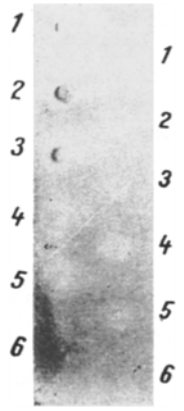

Fig. 2. Comparison of direct and indirect methods of application of MCAB. 1, 2, 3) Rat MCAB to MAFP: D_6, G_4, H_6; 4, 5) rat MCAB to mouse hepatocyte nuclei: B_7, E_7; 6) CM without MCAB. Each reagent applied in a dose of 3 μl: on right — directly to NCM, on left — on dots of mouse MCAB to rat L_K IgG (3 μl). Components of immunologic development of MCAB (in order of their interaction with MCAB): 1) neonatal mouse serum, 1/8; 2) rabbit antiserum to MAFP, 1/10; 3) Fab'-fragments of IgG of a donkey immunized against a rabbit, conjugated with horseradish peroxidase, 1/30. Volume of each reagent 10 μl/cm length of reservoir. After-reaction in 4, 5, and 6 in the left-hand row due to the use of immunoreagents 2 and 3 not exhausted beforehand with embryonic calf serum.

moment when the B_{12} crossed MB the experiment was stopped, the reaction zone was cut out, and peroxidase activity on it was determined. A stained precipitate was found at the site of specific interaction of MCAB to AFB with homologous AG. MCAB of different specificity did not precipitate AFP (Fig. 2).

During work with MCAB in CM, an immunosorbent had to be used. For this purpose, before the beginning of electrophoresis 1-3 μl of CM containing MCAB to MAFP was applied to the rat anti-L_K dots, and B_{12} was applied alongisde. With a voltage of 50 V, MB was carried into the stationary position. B_{12} and unbound MCAB to MAFP were carried by the EEF toward MB. Rat IgG were then applied to the dots so that the free valencies of the rat anti-L_K were blocked. Without blocking in this way, the rat anti-L_K could bind complexes of MAFP with rat MCAB to homologous AG, and thus simulate fixation of MAFP on specific MCAB. B_{12} was applied alongside the site of application of the IgG. As soon as the B_{12} and, consequently, the excess of rat IgG had completely left the reaction zone, the reservoir folds were filled with NMS, anti-MAFP, and Fab'-P respectively. Analysis was conducted in accordance with the scheme described above. The result of comparison of activity of MCAB to MAFP in CM, applied directly to NCM and to the immunosorbent, is shown in Fig. 2 and it illustrates the advantage of the second method. With the aid of the immunosorbent, MCAB were extracted from CM, purified, and concentrated at the point of application, as could be judged by the increase in adsorption of MAFP on them.

Analysis of activity of MCAB to MAFP (D_6, G_4, H_6) and of MCAB to HAFP (D_{10}, D_8, E_3) showed that all MCAB are specific for homologous AG. These data agree with the results of an investigation of these same MCAB, carried out previously by the ELISA method, the mixed precipitation test, and by immunohistochemical methods [3].

The variant of counterflow ITP on NCM, described in this paper, proved to be effective not only for determining activity of MCAB, but also for studying their epitope specificity. The two analyses were conducted on the whole by analogous schemes, the only difference being that for the second method the AG (HAFP or MAFP) were added to R_1 mixed with an excess of one of the MCAB to homologous AFP to be tested (when the immunosorbent — rat anti-L_K — was used, an excess of rat IgG was added to the NMS + MCAB mixture in order to suppress possible competition between MCAB in the mixture and MCAB fixed to the NCM).

AG treated in this way either ceased to be bound by MCAB fixed to NCM or continued to interact specifically with them, to form a precipitate (Fig. 3). In the first case MCAB added

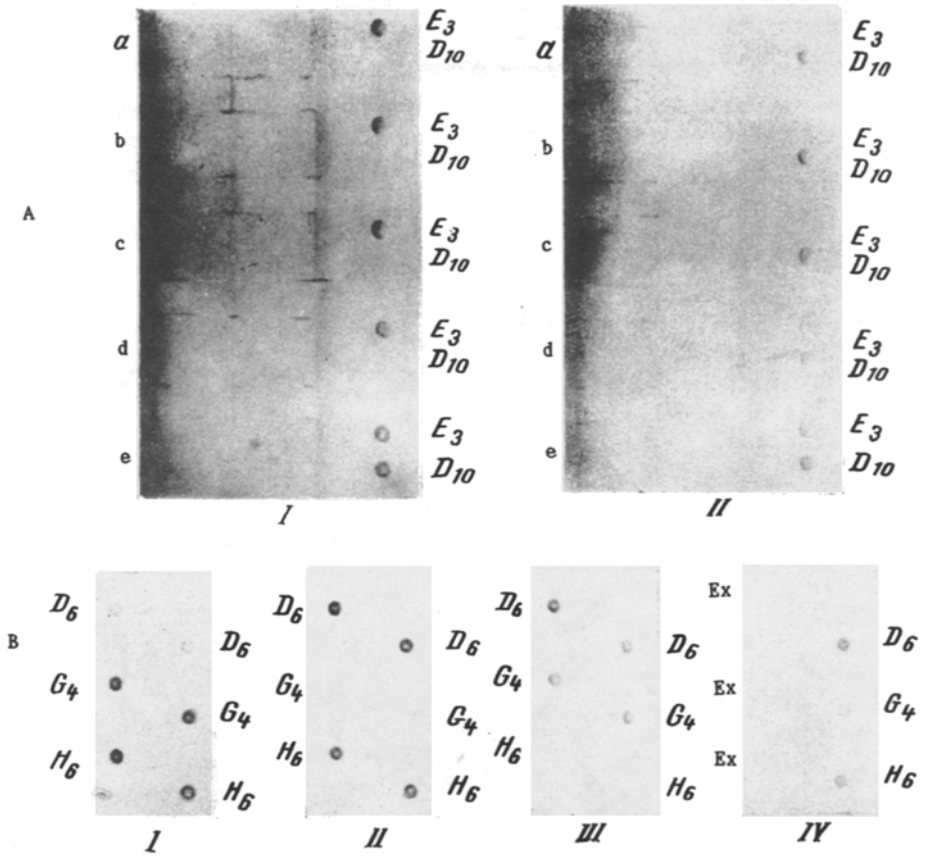

Fig. 3. Analysis of epitope specificity of MCAB to AFP. a) I, II — two strips of NCM. E_3, D_{10}) MCAB to HAFP obtained in ascites fluid. 1 μl applied directly to NCM: each pair of dots lies opposite each of five sections of R_1. Components of immunological development of dots E_3, D_{10}: 1) in five sections (a, b, c, d, e) of NCM I and II — 10 μl of HAFP, 1/16, in each, in four of them (a, b, c, d) mixed with excess of E_3 (NCM I) or D_{10} (NCM II), taken in serial double dilutions (from 1/10 to 1/80), in 5th (on both NCM) — with Tris-HCl, pH 6.7 (control); 2) rabbit antiserum to HAFP, 1/20, 10 μl/cm of R; 3) Fab'-fragments of donkey IgG against rat IgG, conjugated with HRP (Fab'-P), 1/30 — 10 μl/cm of R; b) I, II, III, IV — four strips of NCM. D_6, G_4, H_6 — MCAB to MAFP in CM, applied to immunosorbent — rat anti-L_K; control — immunosorbent without MCAB, blocked with rat IgG. Components of immunological development: 1) MAFP mixed with excess of one of the three MCAB to MAFP on NCM I, II, and III (D_6, G_4, and H_6 respectively). On NCM IV: MAFP without MCAB (control); 2) rabbit antiserum to MAFP 1/8; 3) Fab'-P 1/30. Reagents 2 and 3 were exhausted (Ex), as stated in the text.

to R_1 and MCAB fixed to NCM did not differ in their epitope specificity and competed with each other for the same epitope, whereas in the second case they were not identical with respect to this feature, and for that reason MCAB added to AG in R_1 did not prevent it from interacting with MCAB located on NCM. As a positive control, AG was introduced into one section of R_1 unmixed with MCAB.

An essential preparatory step before epitope analysis is the choice of control in the preliminary experiments. In the final variant, besides the positive control, and internal, negative control, involving AFP mixed with homologous MCAB, must also be used.

This epitope analysis shows that all MCAB to HAFB and MAFB tested have their own individual epitope specificity, in agreement also with the results of an investigation of these MCAB by other methods [3]. By the suggested method it is possible to compare several MCAB preparations quickly in one experiment; moreover, the longest and most laborious operations, namely repeated binding and washing of the immunological reagents, are done virtually without the

involvement of an operator — automatically. It must be emphasized that the method is much easier to carry out in the direct version, i.e., by working with concentrated preparations of antibodies. The direct version is more complex and requires special preparatory work.

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